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High-throughput sequencing reveals suppressors of *Vibrio cholerae* *rpoE* mutations: one fewer porin is enough

Brigid M. Davis* and Matthew K. Waldor

Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School and HHMI, 181 Longwood Avenue, Boston, MA, USA

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ABSTRACT

Analyses of suppressor mutations have been extremely valuable in understanding gene function. However, techniques for mapping suppressor mutations are not available for most bacterial species. Here, we used high-throughput sequencing technology to identify spontaneously arising suppressor mutations that enabled disruption of *rpoE* (which encodes σ^E) in *Vibrio cholerae*, the agent of cholera. The alternative sigma factor σ^E , which is activated by envelope stress, promotes expression of factors that help preserve and/or restore cell envelope integrity. In *Escherichia coli*, *rpoE* is an essential gene that can only be disrupted in the presence of additional suppressor mutations. Among a panel of independent *V. cholerae* *rpoE* mutants, more than 75% contain suppressor mutations that reduce production of OmpU, *V. cholerae*'s principal outer membrane porin. OmpU appears to be a key determinant of *V. cholerae*'s requirement for and production of σ^E . Such dependence upon a single factor contrasts markedly with regulation of σ^E in *E. coli*, in which numerous factors contribute to its activation and none is dominant. We also identified a suppressor mutation that differs from all previously described suppressors in that it elevates, rather than reduces, σ^E 's activity. Finally, analyses of a panel of *rpoE* mutants shed light on the mechanisms by which suppressor mutations may arise in *V. cholerae*.

INTRODUCTION

The alternative sigma factor σ^E (also known as RpoE) is one of several regulators that enable bacteria to respond to perturbation of the cell envelope (1). In unstressed growing cells, σ^E activity is relatively low, as it is tethered

to the bacterial inner membrane by the antisigma factor RseA, and hence cannot interact with RNA polymerase to direct transcription from σ^E -dependent promoters (2,3). However, if misfolded outer membrane proteins (OMPs) accumulate within the periplasm, they trigger the degradation of RseA and release of σ^E , thereby allowing increased expression of the σ^E regulon, which includes genes encoding periplasmic proteases, foldases and chaperones that aid in periplasmic folding, as well as *rpoE* itself (4–6). Activation of σ^E also induces transcription of several small non-coding RNAs (sRNAs) that repress production of OMPs (7). Heightened expression of these factors is thought to aid in elimination of the inducing stimulus and restoration of envelope homeostasis. σ^E activity is also induced as cells enter stationary phase. In *Escherichia coli*, this response has been shown to be dependent upon the alarmone ppGpp, which typically accumulates when nutrients are limited (8,9).

σ^E is classically thought of as regulating a stress response; however, it has been found to be essential for viability in *E. coli* and *Yersinia enterocolitica*, suggesting that it plays a more fundamental role in bacterial physiology as well (10,11). σ^E was not initially recognized to be essential in *E. coli*, both because suppressor mutations can mask this phenotype and because the growth (or lack thereof) of *E. coli* *rpoE* mutants is highly dependent upon the selective criteria employed. However, comparisons of independently derived *E. coli* *rpoE* mutants suggested that these strains might contain suppressor mutations, and several suppressors have been identified to date (12,13). These include a loss of function mutation in *ycdQ*, which encodes a putative DNA-binding protein, and multicopy plasmids containing *ptsN* or *yhbW*. Little is known about the mechanisms by which these suppressors function; however, it has been observed that all the suppressors reduce basal activity of other envelope stress response pathways as well. Somewhat surprisingly, suppressors that counteract *E. coli*'s requirement for the proteases RseP and DegS, which are needed for degradation of RseA and thus for activation of σ^E in response to

*To whom correspondence should be addressed. Tel: 617 525 4655; Fax: 617 525 4660; Email: bdavis@rics.bwh.harvard.edu

periplasmic stresses, did not also function as suppressors for *rpoE* mutations. For example, when production of OmpA and OmpC is reduced, either via deletion of both genes or via overexpression of RseX, an sRNA that negatively regulates them, *degS* and *rseP* are no longer essential; however, *rpoE* is still required (14). This may reflect the fact that several pathways have been shown to activate σ^E in *E. coli*.

Studies of *rpoE* in *V. cholerae*, the etiologic agent of cholera, have not directly addressed whether *rpoE* is essential or whether *rpoE* mutants contain suppressor mutations. As with early studies of *rpoE* in *E. coli*, the ability to generate *rpoE* mutants may have led to the assumption that the gene is not required, without consideration of the possibility of suppressors. In *V. cholerae*, *rpoE* has been shown to be required for intestinal colonization in an animal model of disease and for resistance to an antimicrobial peptide, and likely members of the regulon have been identified (15–17). Furthermore, we have found that expression of σ^E is reduced in the absence of OmpU, an abundant outer membrane protein in *V. cholerae* whose expression is controlled by the key virulence regulator ToxR (18). However, many aspects of the biology of *rpoE* mutants remain to be explored, and phenotypic comparisons of independently derived mutants have not been reported.

Identification of suppressor mutations can be hampered by a lack of scorable/selectable phenotypes and/or of tools for genetic mapping. In *V. cholerae*, there are currently no robust genetic tools for mapping spontaneous suppressor mutations. However, the recent development of massively parallel sequencing technology provides a potential path around these experimental barriers. This technology enables generation of high coverage genomic sequence data at a cost that permits analysis of multiple samples, and hence allows for direct identification of differences between the DNA sequences of related strains. The approach is ideally suited for unbiased identification of genetic changes, particularly if multiple changes with interdependent effects are present within a single strain [e.g. as observed in (19)], since such effects might be missed in phenotypic assays of individual changes.

We generated and characterized a set of *V. cholerae* *rpoE* mutants and found that they displayed a range of phenotypes, consistent with the possibility that they might contain distinct suppressor mutations. Using high-throughput sequencing, we identified several differences between wt and *rpoE* strains that were subsequently confirmed to be suppressor mutations. Remarkably, the majority of strains that lacked σ^E contained additional mutations that reduced expression of *V. cholerae*'s major OMP, OmpU. An *ompU*-independent suppression pathway was also identified, which, unlike other *rpoE* suppressors, elevated the basal activity of this envelope stress response pathway. Overall, our data suggest that a key role for σ^E is to respond to an endogenously generated stimulus transmitted from OmpU. They also shed light on the mechanisms used by *V. cholerae* to maintain appropriate OMP expression, and upon the mechanisms by which suppressor mutations may arise in this organism. Finally, our results illustrate the utility of high-throughput

sequencing technology in carrying out a genomics-based approach to suppressor genetics.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All *V. cholerae* strains generated for this study are derivatives of the sequenced clinical isolate N16961 and are resistant to streptomycin. For analysis of *rpoE* production by *V. cholerae* lacking various OMPs, transposon insertion mutants derived from C6706 were used (20). A related mutant with an insertion in *vca0199* was also used, both with the KnR transposon still present (BD2034) and with the majority of the transposon excised by flippase (BD2041). Transposons were also excised from a subset of the C6706 OMP mutants, namely those in which the transposon 'scar' remaining after excision contained a stop codon to prevent production of the full length gene product. Removal of the transposon, which contains *lacZ*, was necessary prior to monitoring *rpoE::lacZ* activity. C6706 Δ *toxRS* was obtained from JJ Mekalanos. *Escherichia coli* strains DH5 α pir and SM10 λ pir were used for cloning and conjugation of suicide vectors, respectively. Bacteria were grown in LB at 37°C unless otherwise noted, using antibiotics at the following concentrations: streptomycin, 200 μ g/ml; carbenicillin, 50 μ g/ml; kanamycin, 50 μ g/ml. Negative selection against strains containing *sacB* was performed on plates containing 10% sucrose at 30°C. LB plates containing 0.25% SDS were used to assess the sensitivity of *rpoE* mutants to detergents.

Strain and plasmid construction

Strains with insertion mutations in *rpoE* were generated using pBD1574, a derivative of the suicide vector pGP704 that contains 424 bp of an internal fragment of *rpoE* (21). Integration of this vector, via a single crossover event within the *rpoE*-derived sequence, results in gene disruption and confers carbenicillin resistance. Exconjugates were selected on plates containing streptomycin and carbenicillin. When primary exconjugates gave rise to colonies of varied sizes upon restreaking, which occurred for at least 90% of exconjugants, large colonies were selected for further analysis, in order to minimize analyses of mixed populations. Strains with insertion mutations in *ompS* and *ompT* were generated using pBD1664 and pBD1665, which contain internal gene fragments of 451 and 382 bp, respectively.

All allele replacements were generated using vectors derived from the suicide vector pCVD442, using standard allele exchange procedures (22,23). In brief, the targeting vector, which confers carbenicillin resistance, was transferred by conjugation to a *V. cholerae* recipient, where it integrated. Primary exconjugants (selected on plates containing streptomycin and carbenicillin) were then subjected to counterselection on sucrose plates to identify strains that no longer contained the plasmid backbone, and further screened via PCR to identify strains containing the mutation of interest. Strains containing deletions within *rpoE* were generated using pBD1578, which

introduces a 342-bp deletion. Strains in which *rpoE* was replaced by an *rpoE::aph* allele were generated using pBD1999, in which *rpoE* is interrupted by insertion of an *aph* gene cassette from pUC71K. Mutants were selected on plates containing kanamycin as well as sucrose. (Primary selection on plates containing sucrose alone, followed by patching to plates containing kanamycin, led to similar conclusions.) Point mutations in *vca0199*, *vc2413* and the *ompU* (*vc0633*) promoter and 5'UTR were introduced using plasmids pBD2038, pBD2036, pBD2049 and pBD2088, respectively. Deletions in *ompU* and *ompT* were introduced using the previously described vectors pKEK276 and pKEK309 (24). Deletion of *vc0972* was performed using pBD1900.

All *ompU::lacZ* transcription reporter fusions are derivatives of pCB192N, which contains a promoterless *lacZ* cassette (H. Kimsey, manuscript in preparation). Each contains *ompU*-linked sequences extending from position -411 to +227 relative to the *ompU* transcriptional start site (25). The *rpoE::lacZ* transcription reporter fusion is a derivative of pCB192 (26) and contains sequences from -91 to +112 relative to the σ^E dependent transcriptional start site, as defined in (16).

Additional details regarding plasmid and strain construction are available upon request. The sequences of plasmids and the chromosomal loci they were used to alter were confirmed by Sanger sequencing.

Genome sequencing and analysis

Genome sequencing was performed by the Partners Healthcare Center for Genetics and Genomics using an Illumina Genome Analyzer. Sequence data was analyzed using MAQ (<http://maq.sourceforge.net/index.shtml>) (27) and Edena (<http://www.genomic.ch/edena.php>, (28)). MAQ was primarily used for identification of single nucleotide polymorphisms (SNPs), but also enabled detection of some single-nucleotide insertions and deletions in regions with extended mononucleotide tracts. The consensus sequence obtained for *rpoE* mutants was compared to a control sequence from the wt progenitor and to the published *V. cholerae* N16961 sequence, thereby enabling identification both of mutant-specific SNPs and SNPs distinguishing our isolate from the published genome (29). MAQ reports each candidate SNP with a consensus quality score that reflects the trustworthiness of the SNP prediction. Filtered SNPs with consensus quality scores lower than 50 were generally deemed unreliable.

De novo sequence assembly, using Edena, was performed on pooled data from all sequenced samples, using default parameters. Assembled contigs were then compared to the published N16961 genome using MUMmer to identify SNPs, insertions, and deletions (<http://mummer.sourceforge.net>; Supplementary Table 1). Comparable analyses using sequencing results from individual samples did not yield genome-wide results, as the depth of sequence coverage from individual samples was not always sufficient to permit contig assembly; however, a subset of differences could be identified.

The precise locations of sequences that differ between the published N16961 genome and individual *rpoE*

mutants, which were confirmed by Sanger sequencing, are presented in Supplementary Table 2.

β -galactosidase assays

Reporter plasmids were transformed into strains made $\Delta lacZ$ using the plasmid pJL1. Assays were performed basically as described (30).

Western blots

Protein samples were generated from overnight cultures of *V. cholerae* unless otherwise indicated. Culture volumes were normalized based on OD₆₀₀, then cells were pelleted and lysed in 1× NuPAGE LDS sample buffer (Invitrogen) containing 5% β -mercaptoethanol at 95°C. Samples were run on NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes according to the manufacturer's instructions. Membranes were probed with polyclonal antisera to σ^E , OmpU, OmpT, OmpA, or with a commercially available anti 6-His antibody (Genetex). Horseradish peroxidase-conjugated secondary antibodies and Supersignal West Pico chemiluminescent substrate kit (Pierce) were used for detection of bound primary antibodies.

Northern blots

RNA was harvested from log phase cultures unless otherwise noted. RNA was extracted from cell pellets using Trizol (Invitrogen), then treated with DNase I (Qiagen). It was electrophoresed on glyoxal gels (Ambion), then transferred to Bright Star Plus nylon membranes (Ambion). RNA integrity was confirmed via assessment of EtBr-stained rRNA bands. Blots were hybridized to ³²P-labeled *in vitro* transcribed probes in ULTRAhyb (Ambion) at 68°C and washed according to the manufacturer's instructions.

RESULTS

Isolation of *V. cholerae rpoE* mutants

We and others previously isolated *rpoE* mutants of *V. cholerae*; thus, it was clear that there was not an absolute barrier to obtaining *rpoE* mutants in this organism (16,17). However, some of our strategies for generating *rpoE* mutants had failed, while others seemed to have only a low frequency of success. Furthermore, we noted that the two published mutants did not share all phenotypes (e.g. EtOH sensitivity), suggesting that they might have acquired distinct suppressor mutations. Thus, although *V. cholerae rpoE* had not previously been reported to be essential, we undertook a systematic characterization of *V. cholerae rpoE* mutants and their potential suppressor mutations.

We first assessed the frequency of obtaining insertion mutants following conjugation of suicide vectors containing either an internal fragment of *rpoE* or a control gene (*ompS* or *ompT*), along with the selectable marker *bla*. Carbenicillin resistant (*carbR*) mutants of *rpoE*, *ompS* and *ompT* were obtained with approximately equal frequencies, suggesting that these *rpoE* mutants could

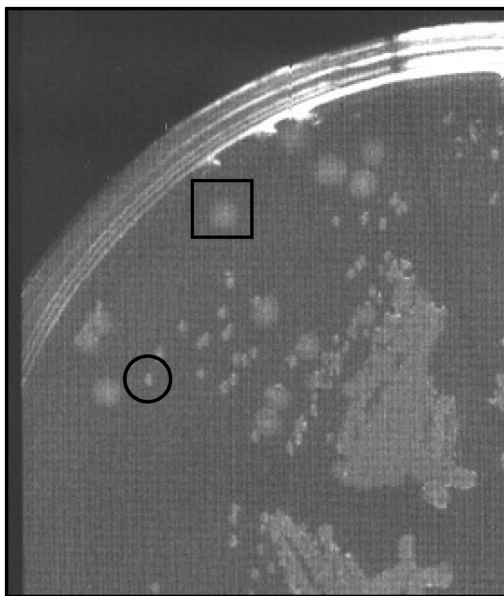


Figure 1. An *rpoE* insertion mutant restreaked on plates containing Sm and Carb following the primary selection. The mutant is a derivative of wt *V. cholerae* N16961. Both large (boxed) and small (circled) colonies are detectable.

arise in the absence of any suppressor mutations. However, we noticed that the initial selection for *rpoE* mutants routinely yielded smaller colonies than for the control genes, and that upon restreaking these colonies typically yielded a mixture of large and small colonies (Figure 1). Further characterization of the mutants revealed that they could be grouped into distinct classes. Mutants could be distinguished by their levels of OMP expression, their colony size and morphology, and their ability to grow on plates containing detergents. Together, these results suggested that our carbR *rpoE* insertion mutants frequently acquired at least one of several additional mutations that suppressed the small colony phenotype of an *rpoE* mutant and enabled more rapid growth.

Derivatives of wt *V. cholerae* in which *rpoE* was deleted, using a targeting vector conferring carbR and sucrose sensitivity, were obtained much less readily than *rpoE* insertion mutants. Identification of deletion mutants required a biased screening of sucrose resistant colonies, namely preferential testing of small or differentially colored colonies. Mutants in which *rpoE* was replaced by *rpoE::aph* could not be obtained. Thus, *rpoE* appeared to be essential for *V. cholerae* growth under the selection conditions for *rpoE::aph*.

Identification of candidate suppressor mutations

To identify the putative suppressor mutations in *V. cholerae rpoE* mutants, we took advantage of newly developed massively parallel sequencing technology. Whole genome sequencing of DNA from a subset of insertion mutants and deletion mutants as well as from wt *V. cholerae* N16961, from which the mutants were derived,

was performed on the Solexa Genome Analyzer. This approach yielded an average of 2.8 million 36-nt sequence reads per sample (range of 1.2–4.0 million). Sequences were subsequently mapped to the published *V. cholerae* N16961 genome sequence (29) using the freely available software MAQ [Mapping and Assembly with Qualities; <http://maq.sourceforge.net/index.shtml> (27)]. MAQ uses the Solexa nucleotide sequences and their associated quality scores (which reflect the confidence with which each nucleotide within a 36-mer was called by the Genome Analyzer) to predict SNPs, positions where the sequenced sample differs from the reference genome. MAQ also calculates its own consensus quality score for these potential SNPs. An average of 88% of the Solexa sequences could be mapped to the reference genome. Numerous potential SNPs were identified; however, most were identified as candidate SNPs in wt as well as *rpoE* mutant samples (and thus correspond to differences between our strain and the published sequence) or had relatively low consensus quality scores. Based on visual inspection of sequence alignments (using MAQ's companion program, MAQview) as well as Sanger sequencing of some candidate loci, we determined that candidate SNPs with consensus quality scores of less than 50 were likely to be false. Removing these loci from the list of mutant-specific candidates left a relatively small number of candidates, suitable for confirmation using Sanger sequencing and/or elimination via visual inspection of aligned reads. Through this process we verified the presence of five candidate suppressor mutations, located in the *vca0199* and *vc2413*-coding regions (one each, in strains BD1965 and BD1963 respectively) and in the intergenic region between *vc0633* and *vc0634* (three distinct mutations, in strains BD1962, BD1956 and BD1987). The effects of these mutations are described below.

Comparison of newly sequenced isolates to the published *V. cholerae* genome

Solexa reads were also used as inputs for the *de novo* assembly program Edena [Exact DE Novo Assembler (28)], which generated contigs that could be searched for differences relative to the published *V. cholerae* N16961 sequence using MUMmer. The advantage of this approach is that it performs gapped as well as ungapped alignments, and hence can be used to identify small insertions and deletions, which generally cannot be detected in our data set using MAQ. Disadvantages are that it does not utilize the Solexa quality scores or provide a means to identify high quality versus low quality candidate SNPs, and it requires a greater number of sequences to generate a consensus, often more than we obtained for a single sample. Edena confirmed the presence of most of the candidate suppressors identified using MAQ; however, its principal utility was in identification of differences between our starting strain and the reference strain, for which we pooled all our Solexa sequences to generate the Edena input. With this comprehensive set of sequences, contigs spanning 3.9 million bases, or >88% of the *V. cholerae* genome, could be assembled, and 145 differences between our strains and the reference

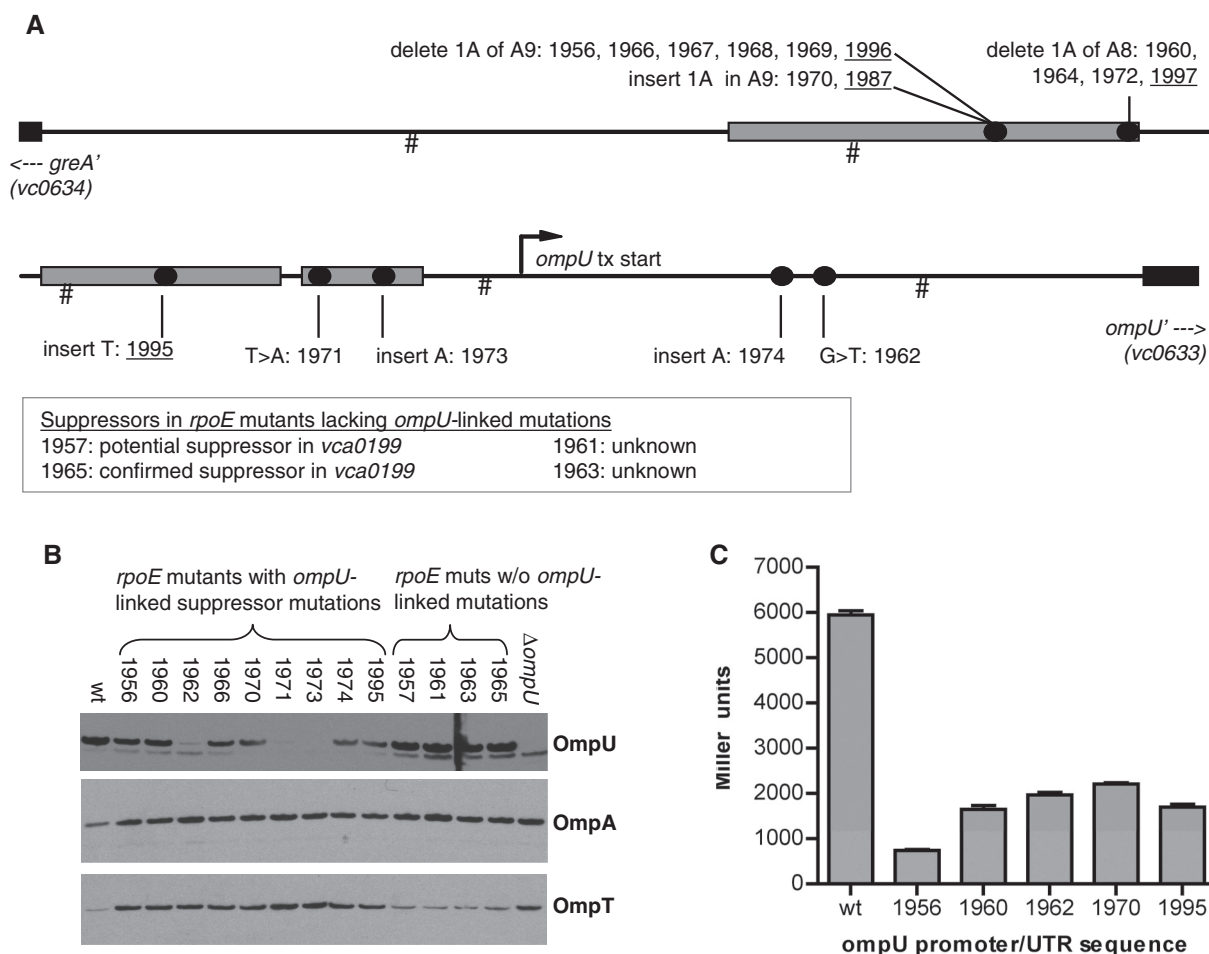


Figure 2. Locations of *rpoE* suppressor mutations and their effects on OMP levels and promoter activity. (A) Schematic depiction of the locations of suppressor mutations in *rpoE* mutants. The *ompU* promoter region is drawn to scale; hash marks denote 100-bp intervals. The 5' ends of the coding sequences of flanking genes are shown as black boxes; the *ompU*-coding sequence corresponds to that shown in (25) rather than the published genome sequence. Binding sites for ToxR are shown as grey rectangles as defined in (32). Circles represent the sites of suppressor mutations, and numbers above (e.g. 1956) correspond to the strain numbers of corresponding *rpoE* mutants. *rpoE* deletion mutants are underlined; all other strains are insertion mutants. In most cases, mutants were independently derived, despite detection of identical suppressors in multiple strains. Only the pairs 1966/1967 and 1968/1969 could each potentially consist of siblings. The precise positions (relative to the published N16961 genome) of all SNPs linked to suppression of *rpoE* mutations are listed in Supplementary Table 2. (B) Western blots showing expression of OmpU, OmpA and OmpT levels in whole cell lysates from wt, $\Delta ompU$, and a non-redundant subset of *rpoE* mutants. (C) β -galactosidase activity from *ompU::lacZ* transcription reporter fusions generated from wt *V. cholerae* and the indicated *rpoE* mutants. Activities shown are averages \pm SEM from three independent cultures and were generated from overnight cultures; log phase cultures yielded similar relative activities.

strain could be identified (Supplementary Table 1). These included 40 SNPs, most of which had also been predicted by MAQ and 79 small insertions or deletions. A subset of these correspond to loci identified in a recent study as likely errors within the published genomic sequence (31). An approximate position for many of the insertions/deletions could also be obtained by performing BLAST analyses on sequences that could not be mapped by MAQ. This approach can be utilized even if sequence depth does not meet the stringent conditions required by Edena, and hence can be used to search for insertions and deletions in sequence from an individual sample as well as pooled samples. However, BLAST analyses did not identify any insertions or deletions in the *rpoE* mutants that were not also present in our wt strain.

Many *rpoE* mutants contain mutations upstream of *ompU* that reduce its expression

The intergenic sequence between *vc0633* and *vc0634*, which was found to contain three independently derived candidate suppressor mutations, contains the promoters for both genes. Notably, *vc0633* encodes OmpU, which we have previously shown is a determinant of σ^E levels (18). Given the frequency of mutations obtained in this region and our knowledge concerning the gene's role, we resequenced this region in all our *rpoE* mutants. Remarkably, these analyses revealed that 17 of 21 *rpoE* mutants contained mutations in this region (Figure 2A). Identical mutations were detected in multiple strains, almost all of which are known to have arisen independently. Most of the mutations are within confirmed binding sites for ToxR, which activates transcription

of *ompU*; however, two of the mutations are not within the promoter, and instead lie within *ompU*'s 5'UTR (21,25,32). *OmpU*-coding sequences were also examined for many of the strains, but no mutations were detected. Western blots showed that all of the strains with *ompU*-linked mutations contained reduced amounts of *OmpU*, although the levels varied widely (Figure 2B). No mutations in this region were identified in the strains found to contain candidate suppressor mutations at loci other than *ompU*, and *OmpU* levels in *rpoE* strains lacking *ompU* mutations were equal to those in wt *V. cholerae*.

To confirm that the *ompU*-linked mutations are responsible for reduced *OmpU* levels, we generated *ompU::lacZ* transcription reporter fusions containing wt and mutant promoter/UTR sequences, and assessed their activity in wt *V. cholerae*. All of the mutant promoter fusions yielded at least 2-fold less activity, as did a reporter fusion containing a mutation within the *ompU* 5'UTR (Figure 2C), suggesting that the mutations do impair *ompU* expression, in most cases presumably due to reduced *ToxR* binding. However, the 5'UTR mutation (identified in strain BD1962) seems likely to influence processes other than, or in addition to, transcription, (e.g. mRNA translation or stability), as its effect on *OmpU* mRNA (data not shown) and protein levels (Figure 2B) was more dramatic than that of promoter mutations that yielded similar reporter activity. Additional northern and β -galactosidase assays suggest that the UTR and promoter mutations do not alter transcription of *vca0634* (data not shown), although the intergenic region presumably contains its promoter as well.

***OmpU*-linked and *vca0199* mutations reduce *V. cholerae*'s requirement for σ^E through distinct processes**

To determine whether the candidate *rpoE* suppressor mutations did in fact facilitate the growth of *rpoE* mutants, we regenerated candidate suppressor mutations in a wt strain, and assessed whether *rpoE::aph* mutants could be obtained in these new strains. Following chromosomal integration of the targeting vector, candidate *rpoE::aph* mutants were selected on plates containing sucrose and kanamycin, and then patched to plates containing carbenicillin to eliminate false positives from the sucrose selection. Using this selection protocol, all sucrose and kanamycin-resistant colonies isolated from the wt strain were also resistant to carbenicillin, i.e. no *rpoE* mutants were obtained (Table 1). In contrast, *rpoE* mutants were readily obtained in strains containing mutations within the *ompU* promoter or 5' UTR, indicating that a reduction in *OmpU* levels obviates *V. cholerae*'s need for σ^E under these selection conditions. A comparable result was observed using strains with deletions of *ompU* or *toxRS* (Table 1). Similarly, we found that *rpoE::aph* mutants were generated at high frequency in the presence of the *vca0199* candidate suppressor mutation. However, no mutants were obtained in a strain containing the *vc2413* candidate suppressor mutation, suggesting that the mutation does not function as a suppressor. The consequences of the *vc2413* mutation were not pursued further.

Table 1. Frequency of obtaining *rpoE::aph* mutants from sucrose/kanamycin selection

Parental strain	Mutation	<i>rpoE</i> mutant ^a	Percentage <i>rpoE::aph</i> (n)
N16961	NA	—	0 (85)
BD2046	<i>vc2413</i>	BD1963	0 (49)
BD2048	<i>vca0199</i> (pt. mut.)	BD1965	91 (102)
BD2064	<i>ompU</i> 5'UTR	BD1962	83 (31)
BD2096	<i>ompU</i> promoter	BD1956	95 (68)
MKW156	Δ <i>ompU</i>	—	96 (46)
JM3-3	Δ <i>ompT</i>	—	0 (33)
BD1902	Δ <i>vc0972</i>	—	0 (45)
BD2139	Δ <i>vca0199</i>	—	45 (65)
C6706	NA	—	0 (48)
BD2041 ^b	<i>vca0199</i> , <i>vca0200</i>	—	0 (52)
BD2093 ^b	Δ <i>toxRS</i>	—	94 (54)

NA, not applicable; n, number of colonies tested for carbenicillin resistance.

^aStrain in which candidate suppressor mutation was initially identified, if applicable.

^bDerived from C6706; all other strains derived from N16961.

Suppressor mutations presumably reduce the cell's need for σ^E ; consequently, it might be expected that production and activation of σ^E would be reduced in strains containing suppressor mutations. Our previous studies of *OmpU* and σ^E suggested that *OmpU* is a key determinant of basal levels of *rpoE* expression, as strains lacking *OmpU* contained markedly lower level of σ^E (18). Mutations that downregulate *OmpU* production (e.g. promoter and 5' UTR mutations) also reduced the abundance of σ^E (Figure 3). In addition, such mutations cause a decrease in σ^E activity, as indicated by reduced β -galactosidase from an *rpoE::lacZ* transcription reporter fusion whose activity is dependent upon σ^E (Figure 4A and data not shown). In contrast, the point mutation in *vca0199* that suppresses *rpoE* essentiality did not appear to influence production of σ^E , and increased σ^E activity several fold, suggesting that it influences survival via a different mechanism (Figures 3 and 4A). Additional attributes of *vca0199* are discussed further below.

Activation of σ^E is growth phase dependent

Although the abundance of *OmpU* is fairly consistent throughout all phases of *V. cholerae* growth, accumulation and activation of σ^E is largely limited to late stationary phase (Figure 4). Cells contain low amounts of σ^E throughout log phase, and σ^E activity in overnight cultures is at least 10-fold higher than in log phase cultures (Figure 4). Similar growth phase-dependent activation in *E. coli* has been attributed to production of ppGpp during stationary phase (8), and ppGpp may account for this activation in *V. cholerae* as well. It should be noted, however, that activation by *OmpU* is not limited to stationary phase; diminished *ompU* expression resulted in reduced activity (relative to the wt strain) in all phases of growth (Figure 4A).

***ompU* mutations modulate *OmpT* levels**

All of the *V. cholerae* *rpoE* mutants contained higher levels of *OmpA* and *OmpT* than were detected in wt *V. cholerae*

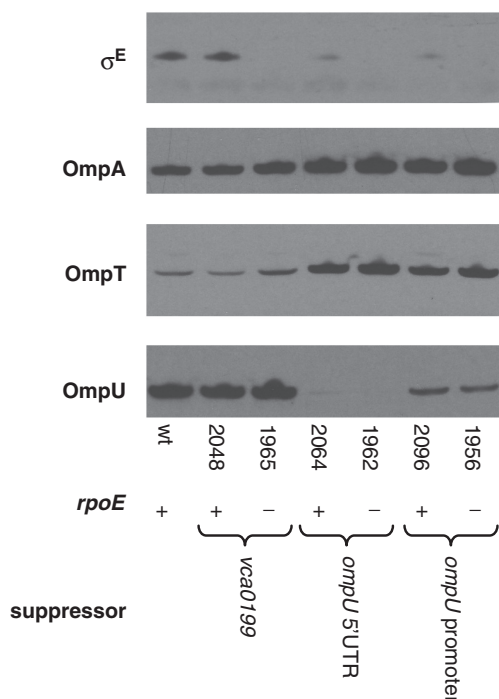


Figure 3. σ^E and OMPs produced by wt *V. cholerae* and strains containing *rpoE* and/or suppressor mutations. Western blots were probed with polyclonal antisera to σ^E , OmpA, OmpT and OmpU. Each *rpoE* mutant (strains BD1965, BD1962 and BD1956) is paired with a strain containing the suppressor mutation (e.g. in *vca0199* or *ompU*-linked sequences) identified within the *rpoE* mutant. Extracts electrophoresed on NuPAGE gels were generated from cells cultured overnight in LB. Culture volumes used for extracts were adjusted based on culture OD₆₀₀ to ensure equal loading.

(Figure 2B). However, unlike the case for OmpA, the degree to which OmpT abundance increased was not uniform among these mutants. OmpT levels were only slightly increased in *rpoE* mutants that lacked *ompU*-linked suppressor mutations, while they were markedly higher in *rpoE* mutants that also contained *ompU*-linked mutations (Figures 2B and 3). These data suggest that σ^E -mediated control of OmpT accumulation [potentially due to σ^E -dependent expression of a regulatory sRNA, as seen for *V. cholerae* OmpA and several *E. coli* OMPs, (33,34)] is relatively minor. Furthermore, they suggest that *ompU* may regulate accumulation of OmpT in a σ^E -independent fashion. Notably, even relatively slight reductions in OmpU had a more significant effect on OmpT accumulation than did disruption of *rpoE* in a strain with normal OmpU production (Figure 3, compare strains BD2096 and BD1965).

OMPs other than OmpU are not major determinants of σ^E abundance and activity in *V. cholerae*

Western blot and *rpoE::lacZ* transcription reporter analyses of a panel of *V. cholerae* OMP mutants suggest that OmpU plays a privileged role with respect to σ^E levels in *V. cholerae*. Strains with mutations in a variety of OMP-encoding genes were obtained from a comprehensive transposon insertion library of *V. cholerae* mutants (20)

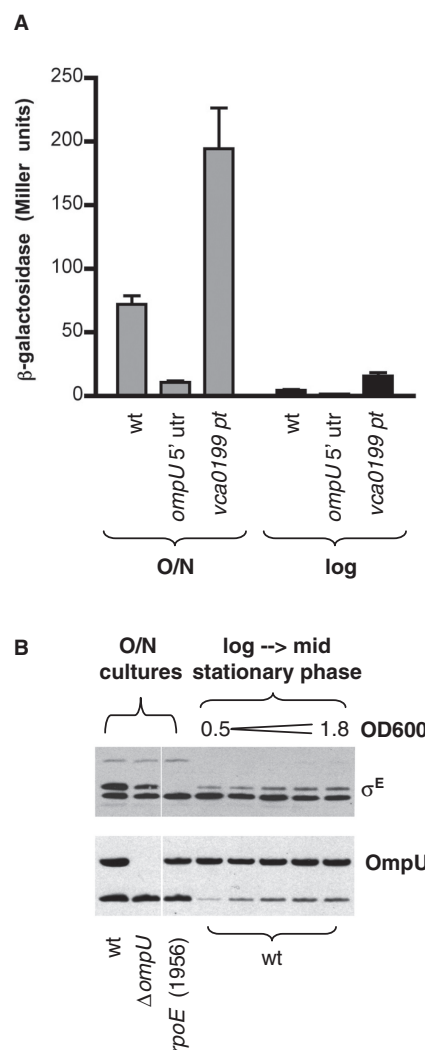


Figure 4. Levels and activity of σ^E and OmpU produced by *V. cholerae* at various growth phases. (A) β -galactosidase activity produced from an *rpoE*-*P2::lacZ* transcription reporter fusion was assessed in a *lacZ* derivative of wt *V. cholerae* (wt) and in strains containing suppressor mutations in *ompU* (BD2064 *lacZ*; *ompU* 5' UTR) and *vca0199* (BD2048 *lacZ*; *vca0199* pt). Activity was assessed in overnight (O/N) and log phase (O.D.₆₀₀ ~ 0.5–0.65; log) cultures. The reporter yielded no activity in a strain lacking *rpoE* (data not shown). Data shown is the mean and SEM for a representative experiment. (B) Western blots of OmpU and σ^E produced by wt *V. cholerae* and the indicated mutants during log and stationary phase growth.

and monitored for production of σ^E . Disruption of *ompU* or of *toxRS*, key activators of *ompU* expression, consistently reduced the abundance of σ^E 2- to 4-fold. In contrast, we found that disruption of genes encoding OMPs other than OmpU generally did not influence σ^E abundance, although disruption of *ompA* and *vc1622* did slightly reduce σ^E levels in a subset of experiments (Figure 5 and data not shown). The cause of inconsistent results with these two mutants is not known. We also monitored the activity of a σ^E -dependent *rpoE::lacZ* transcription reporter fusion in a subset of the OMP mutants (those in which excision of the *lacZ*-encoding transposon resulted in production of a truncated, rather than full

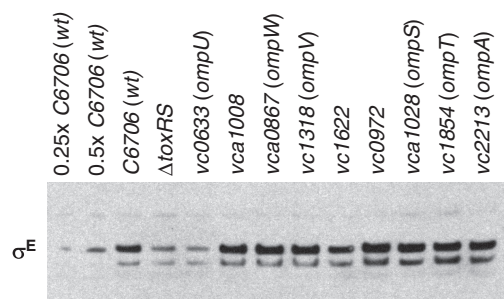


Figure 5. Production of σ^E by *V. cholerae* C6706 and transposon insertion mutants derived from it. The western blot was probed with polyclonal antisera to σ^E . Extracts electrophoresed on NuPAGE gels were generated from cells cultured overnight in LB. Culture volumes used for extracts were adjusted based on culture OD₆₀₀ to ensure equal loading. Disrupted genes encode known and putative outer membrane proteins and porins, plus the positive regulator (ToxRS) of OmpU production. The first two lanes (0.25 \times C6706 and 0.5 \times C6706) were loaded with reduced (1/4 and 1/2) volumes of the wt sample to enable approximate quantitation of signal intensities.

length, OMP). Among this set—which included strains with mutations in *ompA*, *vc1622*, *ompU* and four additional OMP-encoding genes—the activity of the transcription reporter was reduced by more than 2 \times only in the *ompU* mutant (data not shown). Together, these data suggest that, at least for the growth conditions assayed, OmpU is the principal OMP underlying σ^E induction (Figure 5). Deletion of several OMP-encoding genes (*ompT*, *vc0972*) also did not reduce *V. cholerae*'s requirement for σ^E ; *rpoE::aph* mutants could not be obtained in strains lacking these genes (Table 1).

Not all *vca0199* mutations suppress *V. cholerae*'s requirement for *rpoE*

As noted above, a mutation within *vca0199* appears to suppress *V. cholerae*'s requirement for σ^E by a different mechanism than does inhibition of OmpU production, since the *vca0199* mutation increases, rather than diminishes, σ^E activity (Figure 4A). This effect was detectable both in log and stationary phase cultures. *vca0199* does not appear to be a member of the σ^E regulon, based on Northern (data not shown) and previously published microarray analyses (17). Northern blotting also revealed that the suppressor mutation within *vca0199* may cause a small reduction in its transcript and that of the nearby downstream gene, *vca0200*, although such a reduction was not evident in all experiments (Figure 6). *vca0199* and *vca0200* are probably cotranscribed, since an insertion mutation within *vca0199* eliminates transcripts of both genes (Figure 6). We were unable to detect expression of chromosome-encoded epitope-tagged Vca0199 and Vca0200, suggesting that these proteins are probably produced at fairly low levels. However, cell fractionation studies performed using plasmid-encoded Vca0199 with a C-terminal His6 tag indicate that the product of *vca0199* is probably a cytoplasmic protein (data not shown).

No function has yet been described for *vca0199* or *vca0200*, and their putative protein products have strong homology only to hypothetical proteins. The closest

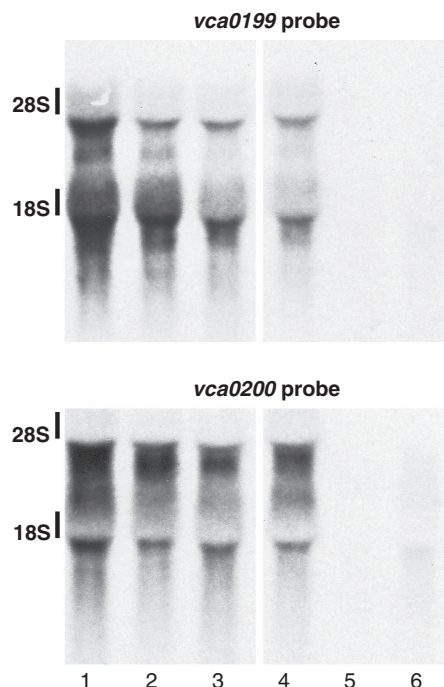


Figure 6. Northern blots probed for transcripts of *vca0199* and *vca0200*. Lanes 1–3 contain RNA from strains derived from N16961; lanes 4–6 contain RNA from strains derived from C6706. Lane 1, wt N16961; lane 2, BD2048 (*vca0199* point mutation); lane 3, BD1965 (*vca0199* point mutation and *rpoE* insertion); lane 4, wt C6706; lane 5, BD2034 (*vca0199* insertion, Kn^R); lane 6, BD2041 (*vca0199* insertion, Kn^S).

homologs are in distantly related organisms, including *Bacteriodes fragilis*, *Listeria innocua* and *Trichodesmium erythraeum*; homologs are absent from vibrios other than *V. cholerae* and from most other gamma-proteobacteria as well, suggesting that these genes were probably acquired relatively recently via horizontal gene transfer. The *vca0199* mutation detected by high-throughput sequencing is predicted to result in substitution of a Y for an S at amino-acid 594 of 638, a region with minimal conservation among *vca0199* homologs. Interestingly, Sanger sequencing of *vca0199* from other *rpoE* mutants lacking *ompU*-linked suppressors identified an additional *vc0199* mutation, predicted to change P616L (in strain BD1957).

Since the effects on protein function of the *vca0199* point mutations described above were not known, we assessed whether additional mutations in *vca0199* could also suppress *V. cholerae*'s requirement for σ^E . Unexpectedly, these assays gave somewhat ambiguous results. An insertion mutation in *vca0199*, which eliminates expression of both *vca0199* and *vca0200*, did not enable replacement of *rpoE* by *rpoE::aph* (Table 1; strain BD2041). In contrast, *rpoE::aph* mutants could be obtained when *vca0199* contained an internal deletion, which should not alter expression of *vca0200* (Table 1, strain BD2139). However, the *rpoE* mutants generated in the latter background grew very slowly; colonies were significantly smaller than those obtained in the presence of the *vca0199* point mutation. Furthermore, mutants were

obtained at a much lower frequency when *vca0199* contained an internal deletion rather than the point mutation. Together, these data suggest that loss of Vca0199 function may facilitate growth of *rpoE* mutants, but only in the presence of Vca0200, and that the consequences of the point mutation are not simply a loss of protein function. Whether the SNP in *vca0199* partially preserves wt protein function and/or results in a novel function remains to be determined.

DISCUSSION

We have assessed *V. cholerae*'s requirement for σ^E and found that it is essential for growth under some but not all selective conditions. Under permissive conditions for disruption of *rpoE*, mutants could readily be obtained but their initial growth was slow. More rapidly growing variants, which arose frequently from the initial isolates, were found to contain additional mutations at unlinked sites. Introduction of these unlinked mutations into wt *V. cholerae* enabled selection of *rpoE* mutants under otherwise non-permissive conditions, confirming that these mutations suppress *V. cholerae*'s need for σ^E . The majority of suppressor mutations reduced production of OmpU, a general diffusion porin that constitutes a significant portion of *V. cholerae*'s outer membrane protein (35,36). The basal level of σ^E declined in strains with *ompU*-linked mutations, presumably reflecting the diminished role for *rpoE* that rendered it non-essential. However, mutations within *vca0199*, which encodes a gene of unknown function, also were found to suppress *V. cholerae*'s need for *rpoE*. Unlike the *ompU*-linked suppressors and suppressors of *rpoE* essentiality identified in *E. coli* (13,37), suppressor mutations within *vca0199* augmented, rather than reduced, σ^E activity, suggesting that they diminish the requirement for σ^E through a distinct mechanism, perhaps by influencing a process downstream of σ^E activation. High-throughput genomic sequencing was a crucial tool in the identification of suppressor mutations, particularly the latter class, which could not have been predicted based on previous knowledge of σ^E 's role and regulation in *V. cholerae*. The availability of this technology, which continues to improve, should greatly facilitate similar studies in other organisms.

We previously observed that deletion of *ompU* reduced *V. cholerae*'s production of σ^E ; however, the importance of this finding was not recognized at the time (18). Our new data highlight the central role played by OmpU as a regulator of σ^E ; indeed, they suggest that one of σ^E 's most critical roles in *V. cholerae* may be to respond to this endogenously generated factor. Even relatively small reductions in OmpU levels have a dramatic effect on the organism's need for, and production of, this sigma factor. It appears a threshold level of OmpU must be present to generate a requirement for σ^E ; simply reducing *ompU* promoter activity by ~60% renders *rpoE* inessential, even though OmpU is still readily detectable. It is also notable that OmpU has a significant influence on activity both during log phase, when much OmpU must be synthesized, and during stationary phase, when OmpU transcript levels

are quite low (data not shown). The latter observation raises the possibility that OmpU's activating stimulus is transmitted from previously synthesized, rather than newly generated, protein. Factors may accumulate in the culture medium by late stationary phase that trigger membrane and OMP disruption and thereby reveal the activating motifs that are masked within properly folded proteins (4). Alternatively, exposure of activating domains within OmpU may be coupled to peptidoglycan remodeling during stationary phase, since OmpU has been reported to be associated with this polymer (36). It is also theoretically possible that the σ^E -activating stimulus is not due to OmpU misfolding but instead is a result of OmpU's porin activity, which might enhance transmission of a signal from the culture media into the periplasmic compartment.

Our study reveals both significant similarities and differences between the role and regulation of σ^E in *V. cholerae* and *E. coli*. For both organisms, *rpoE* is absolutely essential under a subset of conditions tested, and no mutants can be derived from wt cells under these conditions (10). Under less stringent selection conditions, mutants can be isolated; however, they have been found to contain additional suppressor mutations. Such suppressor mutations also permit disruption of *rpoE* under the otherwise non-permissive selective conditions (14,38). Deletion of OMP-encoding genes reduces the activity of σ^E in both *V. cholerae* and *E. coli*. σ^E is also a negative regulator of OMP production in both organisms, although it should be noted that σ^E does not appear to influence *V. cholerae*'s production of its principal OMP, OmpU, since an *rpoE* mutant that lacks an *ompU*-linked suppressor produces wt amounts of this protein (Figure 3), and overexpression of σ^E also did not alter OmpU production (data not shown). A key difference between the species is that no single deletion or combination of deletions of OMP-encoding genes has yet rendered *rpoE* non-essential in *E. coli* (14), whereas the absence of a single *V. cholerae* OMP is sufficient to do so. Thus, it appears that the pathways for activation of σ^E in *V. cholerae* are more limited, and that monitoring and responding to OmpU (mis)folding is of paramount importance. Given that significant activation of σ^E is still detectable in stationary phase cultures of a strain with diminished OmpU, it is quite striking that *rpoE* is no longer essential in these cells.

Our work provides new evidence that bacteria have developed numerous safeguards to coordinate and balance expression of their OMPs. Excessive OMP accumulation was already known to trigger σ^E activity and the resulting transcription of sRNAs that downregulate OMP translation (33,38,39). In addition, it has been noted that OMP-encoding sequences are often adjacent to genes encoding negative regulators of other OMPs, suggesting that new OMP-encoding genes can more readily be acquired in conjunction with means for downregulating those that are already present (40). In this study, we observed that expression of OmpU has a significant negative effect on production of OmpT. It has long been recognized that the genes encoding these proteins respond in opposite fashions to the transcriptional regulator ToxR, and thus that their expression is coordinated (21,32,41); however, disruption of *ompU* has not previously been reported to

augment OmpT production. It remains to be seen whether this interdependence of OmpU and OmpT production is mediated by OmpU's porin function, by an sRNA that overlaps with or is coordinated with the *ompU* transcript, or by some alternate mechanism.

Finally, our identification of a panel of suppressor mutations provides clues about the mechanisms by which such suppressors may arise. Two aspects of these suppressor mutations are particularly striking. First, the same suppressor mutations were generated from distinct selection protocols (i.e. growth in the presence of antibiotics versus sucrose), suggesting that the process for suppressor formation is not determined solely by the selection used. Second, most suppressor mutations (11 of 21) were single nucleotide contractions of mononucleotide (A/T) repeats. In *E. coli*, such mutations are characteristic of the error-prone polymerase DinB, which is induced by specific stresses and is associated with temporary formation of a hypermutable state (42). Although mutation as a stress response has yet to be investigated in *V. cholerae*, our data are consistent with the possibility that formation of *rpoE* suppressor mutations may be facilitated by such a response. Transient emergence of a mutator phenotype may thus underlie the high frequency with which suppressors arise (e.g. Figure 1). Such a possibility could be explored by assessing whether factors often needed for formation of stress-linked mutations (e.g. DinB, RpoS and the SOS response) are also required for development of *rpoE* suppressor mutations. Additionally, blocking these processes might permit characterization of the true consequences of *rpoE* disruption, unmodulated by additional genomic changes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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